Phytochemistry, 1976, Vol 15, pp 137–141 Pergamon Press Printed in England.

IMMUNOLOGICAL COMPARISONS OF HIGHER PLANT PLASTOCYANINS

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(*Received* 5 *June* 1975)

Key Word Index—*Spinacia oleracea*; Chenopodiaceae; *Urtica dioica*; Urticaceae; spinach; nettle; immunology; amino acid sequence correlation; plastocyanin.

Abstract—Antisera were prepared in rabbits to purified plastocyanins of *Spinacia oleracea* and *Urtica dioica*. Using the method of micro-complement fixation, the immunological cross-reactivity of these antisera with plastocyanins from 37 species of plants was determined. Cross-reactivity between antisera to spinach plastocyanin and 11 plastocyanins from other plant species showed a positive correlation with distance on an ancestral amino acid sequence affinity tree constructed by the method of Dayhoff and Eck [1]. The importance of serological data as a supplement to amino acid sequence data in evolutionary studies is discussed.

INTRODUCTION

Serological methods have been applied to plant taxonomic problems since the early part of this century [2], and their use has continued up to the present day [3–5]. In all these investigations a more or less undefined mixture of proteins, usually whole seed extracts, is injected into the experimental animal. The resulting antisera consist of several populations of antibodies, each with its own specificity and avidity. In some cases the cross-reaction data obtained with these antisera appear to cluster taxa in the same way as do non-serological data; that is, the serological results make "taxonomic sense" when classical schemes are taken as the standard [5]. However, other examples can be cited [3] in which the serological affinities cut across taxonomic categories. Such antisystematic results, as Moritz [3] has called them, occur with embarassing frequency, and botanists have tended to treat all serological comparisons with caution.

By contrast, serological studies in the animal kingdom have yielded results which are in agreement with classical taxonomy [6]. This is probably because the antigen employed is blood serum. This is a readily obtainable solution of high and nearly constant protein concentration and consists largely of one highly antigenic protein, serum albumin [7]. When purified serum albumin alone is used as the antigen, there is also a good correspondence between serology and taxonomy [8,9]. This is the case with immunological comparisons using other purified animal proteins as antigens [10–12]. Furthermore, with 3 animal proteins [10,11,13,14] and one bacterial protein [15], the immunological cross-reactivities correlate with amino acid sequence resemblance, which is a direct reflection of gene resemblance.

Since the sequences of 13 higher plant plastocyanins are now known [16–21], and since plastocyanin has been reported to produce antibodies in rabbits [22], it was decided to carry out a serological investigation using plastocyanin as a purified single protein antigen. Although a single protein antigen represents only a single gene, in contrast to protein mixtures, the results using animal proteins indicate that single genes can reflect the evolution of the whole genome. It was anticipated that if antisystematic results were obtained, it would be possible to interpret their cause. Thus, such results would immediately fall into two classes: The first would be that an anomalous serological result was due to the weakness of the correlation between cross-reactivity and sequence resemblance. The second class would be those that correctly assessed plastocyanin sequence resemblance, and would imply a difference between the evolution of the plastocyanin gene and the evolution of the species from which they were derived (again using the classical evolutionary scheme as the standard).

In this paper we report the production of antisera to plastocyanins from *Spinacia oleracea* and from *Urtica dioica* and their cross-reaction with plastocyanins from thirty seven species of higher plants. We show that the magnitude of these cross-reactions is an approximate reflection of plastocyanin gene resemblance, and we compare the phylogenetic inferences with those derived from non-molecular data.

RESULTS

Micro-complement fixation. Table 1 presents the results of micro-complement fixation tests with the two pooled antisera and plastocyanins of thirty seven species of higher plants. The average standard deviation of repeated measurements was 4·2 immunological distance

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Table 1. Immunological differences among higher plant plastocyanins

Source*	Family and Species	Immunological distance from *Urtica* plastocyanin†	Immunological distance from *Spinacia* plastocyanin‡
	Urticaceae		
1. I, II§,¶	1. *Urtica dioica* L.¶§	0	92
2. II	2. *Laportea canadensis* (L.) Gaud. (pp.)¶	40	84
3. III	3. *Pilea pumila* (L.) A. Gray¶	79	62
4.house plant	4. *Pilea nummularifolia* Swartz¶	112	104
	Brassicaceae		
5. IV	5. *Brassica oleracea* var. *capitata* L.§	77	70
6. IV(i)	6. *Brassica rapa* L.¶	86	—
7. I	7. *Capsella bursa-pastoris* (L.) Medic.§	174	73
	Euphorbiaceae		
8. I	8. *Mercurialis perennis* L.§	81	51
9. II	9. *Acalypha rhomboidea* Raf.¶	94	—
	Caprifoliaceae		
10. I	10. *Sambucus nigra* L.¶	94	36
11. II	11. *Sambucus canadensis* L.¶	93	35
	Moraceae		
12. II	12. *Morus alba* L.¶	94	84
	Phytolaccaceae		
13. II	13. *Phytolacca americana* L.¶	98	113
	Cucurbitaceae		
14. IV	14. *Cucurbita pepo* var. *medullosa* L.§	98	50
15. IV(ii)	15. *Cucurbita pepo* var. *torticollis* Alef¶	104	54
	Caryophyllaceae		
16. III	16. *Saponaria officinalis* L.¶	100	83
	Solanaceae		
17. IV(i)	17. *Lycopersicon esculentum* var. *commune* Bailey.¶	102	49
18. IV	18. *Solanum tuberosum* L.§	105	60
	Compositae		
19. IV(ii)	19. *Lactuca sativa* L.¶§	108	66
	Fagaceae		
20. III	20. *Fagus grandifolia* Ehrh.¶	120	46
	Leguminosae		
21. IV(ii)	21. *Phaseolus vulgaris* L.¶	109	96
22. III	22. *Trifolium repens* L.¶	113	57
23. IV(ii)	23. *Phaseolus limensis* Macf.¶	134	126
24. IV(i)	24. *Vicia faba* L.§	153	151
25. III	25. *Albizia julibrissin* Durazz.¶	183	45
	Chenopodiaceae		
26. V	26. *Spinacia oleracea* L.¶	142	0
27. III	27. *Chenopodium murale* L.¶	107	33
28. II	28. *Chenopodium ambrosioides* L.¶	94	34
29. IV(i)	29. *Beta vulgaris* L.¶	>190	101
30. IV(i)	30. *Beta vulgaris* var. *cicla*¶	160	95
	Boraginaceae		
31. IV(ii)	31. *Borago officinalis*¶	108	38
32. I	32. *Symphytum officinale* L.§	132	33
	Polygonaceae		
33. I	33. *Rumex obtusifolius* L.§	127	112
34. III	34. *Polygonum caespitosum* var. *longisetum* Blume	124	95
	Menyanthaceae¶		
35. I	35. *Nymphoides peltatum* (S. G. Gmel) Kuntze§	192	142
	Umbelliferae		
36. I	36. *Aegopodium podagraria* L.§	184	167
	Basellaceae		
37. IV(ii)	37. *Basella alba*¶	170	101

* Source of specimens; I, County Durham, England; II, Montgomery County, Maryland, U.S.A.; III, District of Columbia, U.S.A.; IV, grown from commercial seed: (i) Ferry-Morse Seed Company, Mountain View, California; (ii) W. A. Burpee Co., Philadelphia, Pa., U.S.A.; V, local market, California, U.S.A. † Cross-reactions were carried out with a pool of 3 antisera to *Urtica* plastocyanin, N2,N3,N4 (2·1:1·0:3·1), whose micro-complement fixation pool titer is 1/2000. Micro-complement fixation titer is defined as the antiserum dilution required to obtain a reaction curve with peak height of 75% complement fixed. The immunization period was 43 weeks. ‡ Cross-reactions carried out with a pool of 2 antisera to *Spinacia* plastocyanin, S3013, S3015 (11·5:1), whose micro-complement fixation pool titer was 1/720, immunization period, 1 yr. ¶ Crude antigen. § Pure antigen.

units for reactions with antisera to nettle plastocyanin, and 3·4 units for antisera to spinach plastocyanin.

Consider first the cross-reactions to anti-nettle (*Urtica*). The plastocyanin of *Laportea canadensis*, which is in the same family as nettle, is immunologically similar to that of nettle. Plastocyanins from representatives of other flowering plant families are more remote, and one, from *Beta vulgaris*, is too dissimilar to nettle to react. Among the flowering plants, the taxonomic unit which has seemed to be the most firmly established is the family. Therefore, it is of particular interest to see if immunological comparisons of a single protein, such as plastocyanin, will reflect the morphologically-established integrity of this unit. If this is so, one would expect members of a particular family to appear as a discrete cluster, all equi-distant from nettle (or from spinach). Table 1 shows clearly that this is not the case. For example, the plastocyanin of *Brassica oleracea* gave a moderately strong reaction with anti-nettle, immunological distance = 77, but that of *Capsella bursa-pastoris*, which is also a member of the Brassicaceae, reacted weakly, immunological distance = 174. Similar results were observed for members of the Leguminosae and Chenopodiaceae. Even among the Urticaceae, *Pilea nummularifolia* was more remote from nettle than were several species outside the nettle family. At a higher level, we can examine the data for evidence of ordinal clustering. *Morus alba* and *Fagus grandifolia* are considered to belong to the same order as nettle [23] (Urticales) but their plastocyanins were no closer to nettle plastocyanins than were those of several other families (Brassicaceae and Euphorbiaceae), which are not morphologically related to nettle.

The antiserum pool directed towards spinach plastocyanin gave results that complement and supplement those obtained with anti-nettle. The diversity of reaction of family members is seen again in the Leguminosae and Chenopodiaceae, but the perspective is different. In the case of the Chenopodiaceae, the plastocyanin of *Beta vulgaris* appears to be quite unrelated to those of spinach, but those of two *Chenopodium* species (*C. murale* and *C. ambrosioides*) are closely related. Also, spinach is closer to *Pilea pumila* and *Pilea nummularifolia* than nettle. As with anti-nettle, no ordinal clustering is evident; members of families which are included in the Centrospermae, such as *Phytolacca americana* or *Saponaria officinalis* [23], do not show strong plastocyanin affinities to spinach. By contrast, representatives of supposedly unrelated families, such as *Sambucus nigra* and *S. canadensis* and *Symphytum officinale*, are as close by these immunological tests as are the *Chenopodium* species. Finally, *Rumex obtusifolius* and *Polygonum caespitosum*, members of the Polygonaceae, have often been considered to have affinities with the Chenopodiaceae [24]; whereas phytochemical studies [25] have placed these two taxa in more widely separated groups. The plastocyanin comparisons support the latter arrangement.

Correlation of immunological cross-reactivity and the degree of sequence resemblance.

Figure 1 shows the correlation between per cent amino acid sequence difference and immunological distance for both anti-spinach and anti-nettle cross-reactions. The correlation is $r = +0{\cdot}67$, somewhat lower than that observed for animal cytochromes *c* [10,26]. The correlation can also be described by the equation $Y = AX + B$,

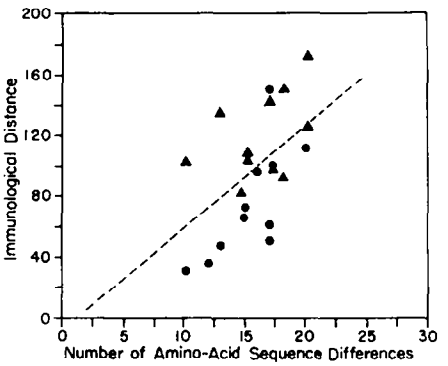

Fig. 1. Correlation of immunological cross-reactivity with degree of sequence difference for anti-plastocyanin sera. Cross-reactions were carried out using the method of quantitative micro-complement fixation. The antiserum pools directed to nettle (▲) and to spinach (●) were identical to the ones described in Table 1. Additional details are given in Wallace and Boulter [26].

where Y is expressed in immunological distance units, X is the % sequence difference, and $A = 6{\cdot}7$, $B = -8$. The 95% confidence limits for the constants are 10·1 and 3·3 for A and 6 and -22 for B. If anti-spinach and anti-nettle pools are considered separately, the linear correlation coefficients and regression equations are $r = +0{\cdot}76$, $Y = 5{\cdot}9X - 15$; and $r = +0{\cdot}82$, $Y = 6{\cdot}5X + 13$, respectively. The 95% confidence limits are similar. The results from both pools are plotted together to emphasize the fact that the approximate relation $Y \simeq 5X$ is observed for these antisera, as is the case for antisera, as is the case for antisera to avian lysozymes [13] and bacterial azurins [15].

It should be noted that the cross-reactions (and correlations) observed are dependent upon the composition of the antiserum pools. The rationale for pooling is to equalize the reactivity of antisera from different rabbits. For the purposes of a study such as this, what is desired is a population of antibodies which gives the best possible correlation between immunological cross-reactivity and sequence difference. Whether this goal can be achieved by using a single antiserum or an antiserum pool must be determined empirically. With small antigens, such as plastocyanin, the reactivities of antisera from different rabbits may vary greatly. For example, an additional antiserum to nettle, (N5) derived from a fourth rabbit, gave a poor sequence–immunology correlation when tested alone ($r_{N5} = +0{\cdot}41$) or in a pool with the other three antisera ($r_{N2,N3,N4,N5} = +0{\cdot}53$). Therefore, this antiserum was not used in determining the cross-reactions of Table 1. or Figs. 1 and 2. Additional details concerning antiserum pooling are given elsewhere [13,26].

The correlation between immunological cross-reactivity and affinity distance on ancestral amino acid sequence tree

In order to use immunological results to supplement phylogenies based on sequence data, it is first necessary to show that immunological cross-reactions correlate with affinity tree distances. Phylogenetic trees may be constructed using matrix methods [27] or by using amino acid ancestral sequence methods [1,28]. Using the

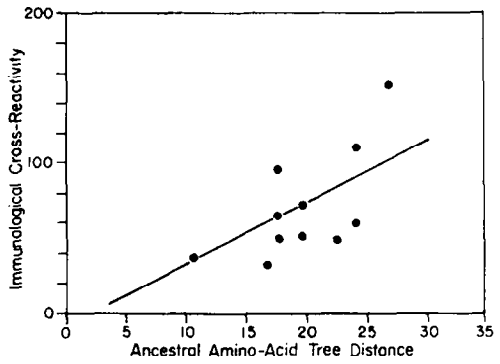

Fig. 2. Correlation of immunological cross-reactivity with ancestral sequence tree distance. Immunological cross-reactions were carried out with the anti-spinach plastocyanin pool of Table 1 and eleven plastocyanins (in addition to the reference species, spinach plastocyanin). In order of increasing immunological distance, the plastocyanins were from: *Symphytum officinale*, *Sambucus nigra*, *Lycopersicon esculentum*, *Cucurbita pepo*, *Mercurialis perennis*, *Solanum tuberosum*, *Lactuca sativa*, *Capsella bursa-pastoris*, *Phaseolus vulgaris*, *Rumex obtusifolius* and *Vicia faba*.

data from an ancestral amino acid sequence tree constructed from twelve higher plant plastocyanins, Figure 2 is a plot of immunological distance *vs* tree distance, with spinach plastocyanin as the reference protein. The correlation obtained with spinach cross-reactivities was +0·78; significant at greater than the 99% level. It was not possible to make the comparison with nettle as a reference protein, since, when the nettle sequence is included in the data set, a unique ancestral sequence tree cannot be constructed. When more plastocyanin sequences are available, the nettle position on the tree will presumably stabilize [24], and then a correlation can be tested. By comparison with the present findings, the correlation obtained with animal cytochromes *c* is $r = +0{\cdot}82$ (log % homology by macro-complement fixation assay vs tree distance, 34 comparisons, significant at greater than the 99·9% level), using the immunological measurements of Margoliash *et al.* [10] and the ancestral sequence tree of Dayhoff [28].

DISCUSSION

The object of the present investigation was to see, firstly, if using single protein antigens a correlation exists between immunological cross-reactivity and sequence resemblance, and, secondly, whether this correlation extends to ancestral sequence tree distance. Both correlations have indeed been established. This means that phylogenetic inferences derived from our immunological comparisons should resemble, to the extent described by the correlation, those derived from sequence data alone. In addition, if one prepares antisera to plastocyanins from additional plant species (10 or more would be desirable), phylogenies can be constructed completely from immunological data [29].

However, we have already seen that the results of several cross-reaction tests are in striking disagreement with classical taxonomic groupings. In view of the spread of points in Fig. 1, the first question to consider is whether these antisystematic results are indeed reflected in plastocyanin sequence differences. In three of the most extreme cases we have a direct comparison: (1) The large immunological distance between *Beta* and spinach plastocyanins, whilst unexpected from classical taxonomic treatment, is reflected in the sequence difference of about 20% between these two proteins [30] (2) The large immunological distance between the plastocyanins of *Vicia faba* and *Phaseolus vulgaris* also correlates with a 20% difference in their sequences [17], and (3) the immunological closeness of *Symphytum* and *Sambucus nigra* to spinach, whilst taxonomically surprising, is in keeping with the sequence differences, which are 10 and 12%, respectively [26].

Because of the correlation of immunology and sequence difference with ancestral tree distance, we would then expect the ancestral sequence tree to reflect these antisystematic features, as well. Thus, we cannot escape the conclusion that for the plastocyanin gene, affinities between members of different plant families are often greater than between members of the same plant family.

Given that the plastocyanin gene apparently does not follow the classical treatment, how does it compare with cytochrome *c*? Although a complete answer cannot at present be given, due to an inadequate number of plastocyanin sequences, it does appear that the plastocyanin sequence tree is about the same as that for cytochrome *c* [18,31]. In the case of cytochrome *c* there is as yet no evidence that the plant families are scrambled, but no more than three representatives of any one family have been examined.

If it is shown that plastocyanin differs from cytochrome *c* in this respect, it would suggest different selection pressures upon the chloroplast relative to the mitochondrion. The higher plant families are very old, greater than 1×10^8 million ys [32] and highly disparate rates of evolution after family divergence could create a situation in which each family contains some "primitive" and some "advanced" members with respect to plastocyanin. The members with primitive plastocyanins would still show moderate resemblance to primitive members of other families; for example, the plastocyanins of *Urtica* and *Brassica*. Advanced plastocyanins, such as that of *Vicia faba*, would have evolved away from members of their own family and probably from other families, as well. Such large differences in evolutionary rates over such long times have not often been observed in the animal kingdom [28], but plants, since they are not mobile, may have to modify some aspects of their metabolism more drastically in response to environmental change.

Finally, it cannot be completely discounted that some of these antisystematic results have arisen because the classical taxonomic scheme is not the best treatment of the taxa involved. The classical schemes are the result of comparing numerous morphological characters which presumably reflect the evolution of many loci on the genome. However, most of these characters cannot be measured quantitatively and appear to have arisen several times in the course of plant evolution. Therefore, in the absence of an adequate fossil record, there is no reason *a priori* to consider these characters to be superior to molecular ones.

This paper has shown that the use of a single protein antigen for serological studies can describe affinities within one gene in higher plants. Furthermore, because the system is defined, anomalies can be identified and

taken into account [26]. Nevertheless, in spite of these presumed advantages over the traditional serological methods, the results obtained are still not in agreement with classical taxonomic and phylogenetic treatments.

EXPERIMENTAL

Plastocyanin was isolated from green leaves of 37 species of plants and purified either completely or in part, as described by Wallace and Boulter [26] and Ramshaw *et al.* [33]. Plants collected in the field in Britain were identified by Mr. D. Sayers, Univ. of Durham, Botany Dept. and those collected in the U.S., by Dr. J. J. Wurdack, Smithsonian Institution, Museum of Natural History, Washington, D.C. Voucher specimens were retained in the Department of Botany, University of Durham. Antibodies were prepared in rabbits to purified plastocyanins of *Spinacia oleracea* and *Urtica dioica*.

Immunological cross-reactions were measured using the quantitative micro-complement fixation technique [34], and results expressed in units of immunological distance. The immunological distance between two identical plastocyanins is zero, whereas very distantly related or unrelated plastocyanins have immunological distances greater than 200. Antisera gave the same cross-reaction with both crude and purified plastocyanins of the same species and therefore, in micro-complement fixation tests, the antisera were directed exclusively to plastocyanins and not to impurities [26]. Anti-spinach sera from two rabbits and anti-stinging nettle sera from three rabbits were used, and for each species antisera from the different rabbits were pooled in inverse proportion to their micro-complement fixing titer. An additional antiserum to stinging nettle, derived from a fourth rabbit, gave a poor sequence-immunology correlation, and it was not used in this study. Details of these methods are described in Wallace and Boulter [26].

Acknowledgements—We are grateful to the University of Durham Zoology Department and to the Department of Biochemistry, University of California, Berkeley, for providing facilities for housing rabbits. We thank Drs. K. Bowler, G. Cocks and J. Liddell for assistance in immunizing rabbits, and Drs. R. Brown, R. Malkin, J. A. M. Ramshaw and M. D. Scawen for providing some of the purified plastocyanins. We also thank Drs. S. Carlson, J. A. M. Ramshaw, V. M. Sarich and M. D. Scawen for shipping antisera; Dr. D. Peacock for data handling, and Mr. D. Sayers and Dr. J. J. Wurdack for checking the identity of plant specimens. Excellent technical assistance was provided by Miss L. Gazze, Mr. J. Gilroy, Mr. P. Hunter and Mrs N. Van de Velde. Finally, we thank L. Gottlieb, P. Haas, M. Mersky, R. Lande and J. A. M. Ramshaw for advice and discussion. This work was supported in part by a Leverhulme Trust Fellowship to D.G.W. and by N.S.F. Grant No. GB-27585.

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